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14. ABSTRACT <p>Mutational events that drive a normal cell to become a cancer cell require the coordinated overexpression of multiple biomarkers. Unique biomarker combinations can create dynamic, physiologic patterns at different stages of cancer development. The assignment of protein expression patterns, or protein profiling, to delineate differences between normal tissues and developing cancer is gaining momentum as a critical instrument in aiding diagnosis, tailoring therapeutics, and predicting clinical outcomes. These proteomic studies, however, investigate changes in protein expression in cell lines and bulk tissue specimens at the gross proteomic level. While powerful, this technique fails to account for the heterogeneity of most tumors since the histopathology associated with many cancers encompasses only a small fraction of the total number of cells present in a tissue section. To date, it is impossible to visualize these cancer biomarker patterns <i>in situ</i>, which define the status of the cell, both non-invasively and <i>in vivo</i>.</p> <p>We present a major leap in cancer imaging ideology to develop a novel molecular imaging paradigm that utilizes multiple cellular targets to generate imageable signals in the same cell thereby achieving chemical resolution. <i>The objective of our proposed research is to develop a new imaging platform consisting of targeted-trans-complementing reporter fragments to simultaneously image the cancer signature in vivo and in real time.</i> A number of unique advantages of this imaging platform are envisaged, such as: 1) increased specificity; 2) multi-marker imaging at the cellular level; and 3) ability to recognize distinct genetic patterns among different tumor types and grades. In the proposed experiments, we will conjugate each reporter fragment to one of two different ligands to simultaneously target and image <i>in vivo</i>, via enzyme reporter re-construction in situ, a molecular signature consisting of two or more different targets. If the cells contain only one or none of the targets, no reporter signal will be observed. The feasibility of this imaging paradigm will be conducted in vivo with animals bearing tumors that express either a subset or all of the biomarkers required for enzyme <i>trans</i>-complementation.</p>					
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INTRODUCTION

Targeted-reporter platforms answer a critical unmet need and have real application for imaging the multi-step progression of cancer growth that requires the coordinated overexpression of multiple biomarkers. The development of these platforms to investigate molecular signatures associated with disease creates the next frontier in *in vivo* imaging. The importance for imaging molecular signatures is underscored by the almost weekly publication of sets of genomic markers that are diagnostic or predictive of disease states. By exploiting multi-marker imaging, we ultimately seek to image combinations of biomarkers that will uniquely identify cancers from normal tissue and report on the biochemical status of cancer cells. These expression patterns can thus be indicative of the type, stage, or severity of disease. However, the application of imaging molecular signatures is far more expansive than just applications for cancer diagnosis or disease detection. Once developed, we will be able to transform molecular imaging from single biomarker identification to imaging and interrogating cellular dynamics during other complex processes such as embryonic development, growth, proliferation, and differentiation in addition to cellular changes that occur early in the course of disease.

Our research seeks to utilize relatively innocuous building blocks to create an exquisitely intuitive platform. By linking a reporter fragment to a targeting moiety, we are only constrained by the number of fragments with which the reporter can be cleaved. The underlying hypothesis of this work is: trans-complementing subunits of image-able enzymes can be exploited to design a system so that multiple changes in a cancer cell's diagnostic signature can be imaged simultaneously. This approach is only achievable by utilizing subunit complementation, which provides a chemical resolution that is far more precise than the physical or anatomical-based resolution currently employed to produce data using non-invasive imaging hardware. The pioneering utility of the technique significantly increases specificity, decreases background artifacts, and promotes our ability to interrogate the status of cells rather than just the presence of cancer biomarkers. Thus, by expanding the number of biomarkers available to non-invasive diagnostic and therapeutic assessment, we envision an innovative platform-based approach to disease identification, staging, and treatment.

BODY

Our research aims to utilize inactive subunits of an image-able enzyme that are added exogenously to the targeted cells or tissue via intravenous injection into the animal. No tinkering with the genome of the diseased tissue is required and these bio-probes can be utilized to report on the physiologic expression levels of important biomarkers in any tissue to which they are administered. The development of such a non-invasive imaging system that bases disease detection on biochemical differences rather than on anatomical differences between normal and diseased tissues is clearly innovative and provides an intelligent-design strategy to pursue non-invasive assessment of diagnostic and prognostic genetic signatures *in vivo*. Eventually, this technology will have the ability to accurately assess the entire mass of a diseased tissue rather than a limited biopsy of the tissue, providing a more comprehensive knowledge of genetic changes than either individual genetic or proteomic analyses can achieve.

To develop an epidermal growth factor receptor (EGFR) targeted imaging complex that would cross the Blood Brain Barrier (BBB) and selectively bind to brain tumor cells overexpressing EGFR, we created a peptide-based near infrared (NIRF) probe to compare to full-length EGF. This allowed us to assay specificity, kinetic behavior, and binding affinity of the receptor-targeted peptides as outlined in **Milestone 1**. For these studies, we employed a peptide discovered through phage display screening against purified human EGFR. The peptide was modified to include linkers and a NIRF dye (**Fig. 1**). To

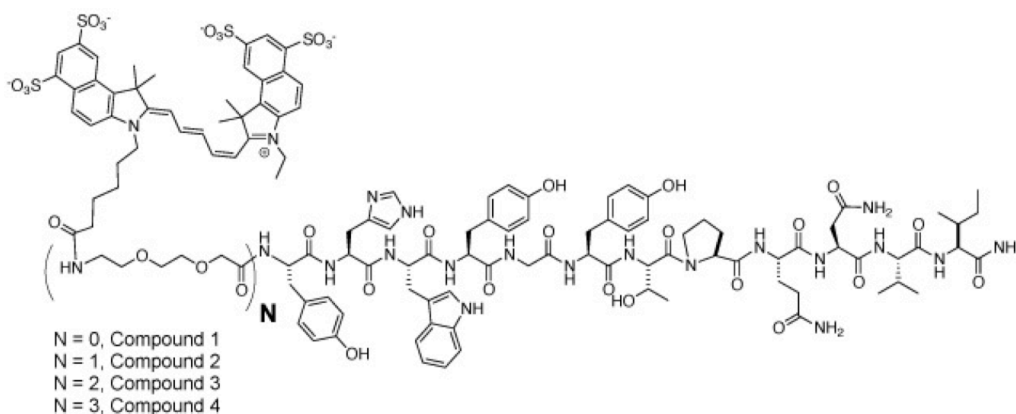


Fig. 1. EGFR-binding peptides (EGF_{peg}). EGF_{peg} is conjugated to a near-infrared fluorophore, Cy5.5, linked with up to three units of amino-ethoxy-ethoxy-acid (AEEA) at the N-terminal amine of the peptide.

determine the optimal space between the NIRF dye and the peptide, we designed and synthesized a series of peptides to include increasing numbers of discrete ethylene glycol units to serve as linkers between a Cy5.5 and the N-terminal end of the peptide. Cy5.5 and EGF_{pep} were either directly linked or linked via 1, 2, or 3 units of discrete ethylene glycol (AEEA) moieties (**Fig. 1**). To determine which of the compounds optimally interacts with cells expressing EGFR, the apparent binding for each bioconjugate was fluorometrically determined from a saturation binding assay in vitro using a human GBM cell line overexpressing EGFR, Gli36Δ5, (**Table 1**). Compounds 1, 2, 3, and 4 all bound to the cells with affinities in the micromolar (μM) range. Compound 2, which had a single linker, had the highest apparent affinity with a K_d at least 2-fold better than compound 1, which had no ethylene linker, 8.9 μM to 18.5 μM respectively. Compounds 3 and 4 have significantly higher affinities for binding, 64.4 μM and 123.0 μM, respectively.

Compound	Peptide (EGF _{pep})	K _d (μM)
1	Cy5.5-YHWYGYTPQNVI-amide	18.5 ± 3.9
2	Cy5.5-(AEEA) ₁ -YHWYGYTPQNVI-amide	8.9 ± 3.7
3	Cy5.5-(AEEA) ₂ -YHWYGYTPQNVI-amide	64.4 ± 24.6
4	Cy5.5-(AEEA) ₃ -YHWYGYTPQNVI-amide	123.0 ± 174.0
5	YHWYGYTPQNVI-amide (GE11-amide)	ND
6	Cy5.5-(AEEA) ₁ -NYQTPVYGWYH-amide (scrambled)	ND

Table 1. Binding affinities of the EGF peptides.

We next used immunofluorescence microscopy to determine the fate of the peptide complexes once they bound to glioblastoma cells expressing EGFR. As predicted from the affinity measurements, compound 2, which had the highest affinity, also showed the greatest accumulation of fluorescence after incubation with Gli36Δ5 cells (**Fig. 2A**). Neither compound 1 (no linker) nor either of the molecules with greater linker numbers (compounds 3 and 4) was taken up by the cells to the same extent as compound 2. Interestingly, cells took up the peptide with the longest linker and the worst binding affinity, compound 4, better than compound 3 (**Fig. 2B**). Compound 4 compares most closely to commercially available full-length EGF by linker numbers; however, the presence of the hydrophilic Cy5.5 fluorophore appears to impact binding affinity. When tested against U87-MG cells, which express much lower levels of the EGFR (**Fig. 4A**), no cellular uptake for any of the compounds was observed (**Fig. 3**).

Since we wanted to specifically target wild-type EGFR and not mutant derivatives, such as the EGFRvIII, often found in cancer and a different cancer cell lines, we also examined the expression of EGFRvIII in various cell lines. Western blots for the EGFRvIII on both U87-MG and Gli36Δ5 cells showed that the mutant receptor expression is similar (**Fig. 4B**) for both cell lines. In addition, saturation binding studies conducted with A431 cells, a squamous carcinoma cell line, that expresses high levels of only wild type EGFR (**Fig. 5A**), indicated that the K_d for binding was similar to that measured with

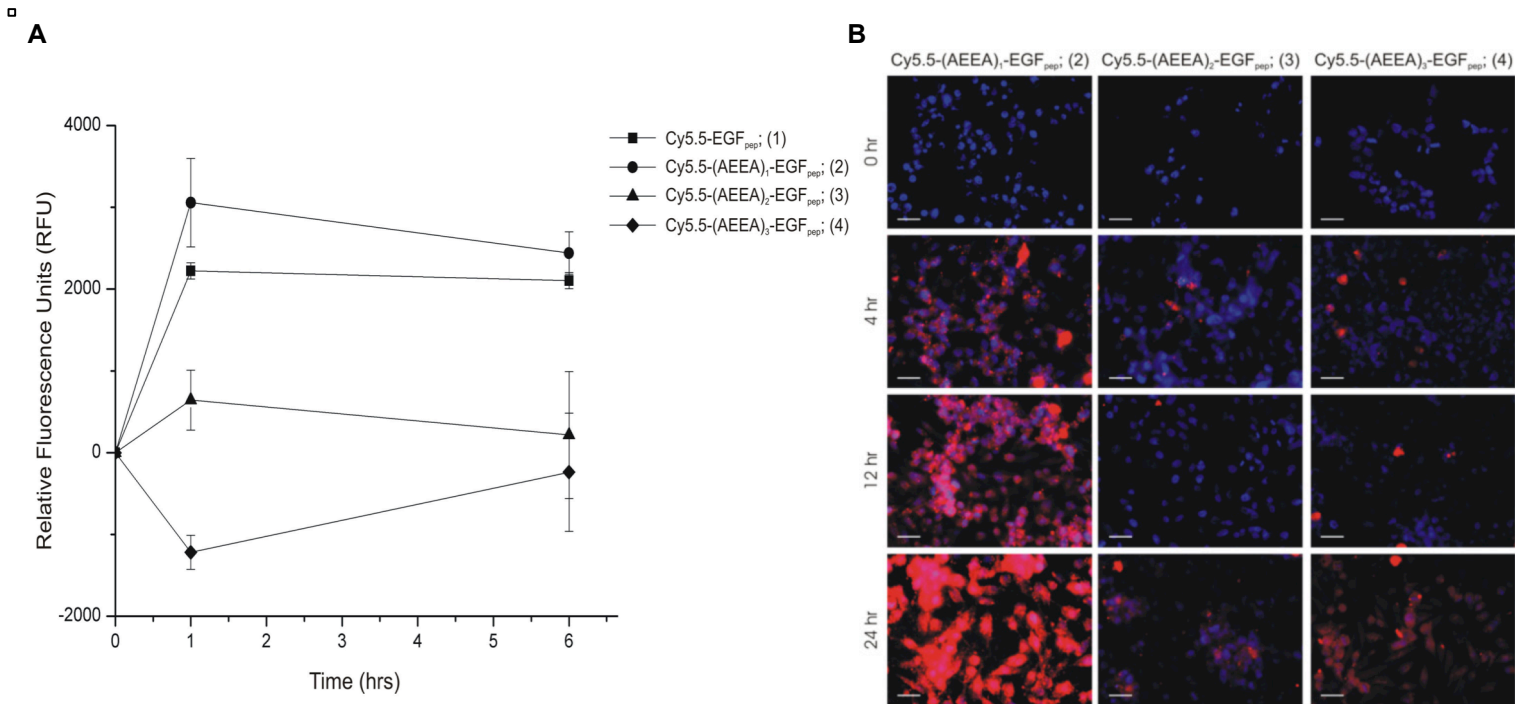


Fig. 2. *In vitro* studies of the peptides with different linker lengths. **A**, Gli36Δ5 cells were incubated with 1 μM of compound 2 [Cy5.5-(AEEA)₁-EGF_{pep}] and the cell associated fluorescence was measured using spectrofluorometry at the indicated times. **B**, Cells were incubated with 1 μM compounds over time. Uptake of the compounds was assessed using epi-fluorescence microscopy. Representative images are shown. Images = 40X magnification. Scale bar = 50 μm.

Gli36Δ5 cells (**Fig. 5B**). Further, incubation of compound **2** with A431 cells displayed increasing fluorescence in the presence of the EGF ligand, which increases cycling of wild type EGFR in cells (**Fig. 5C**).

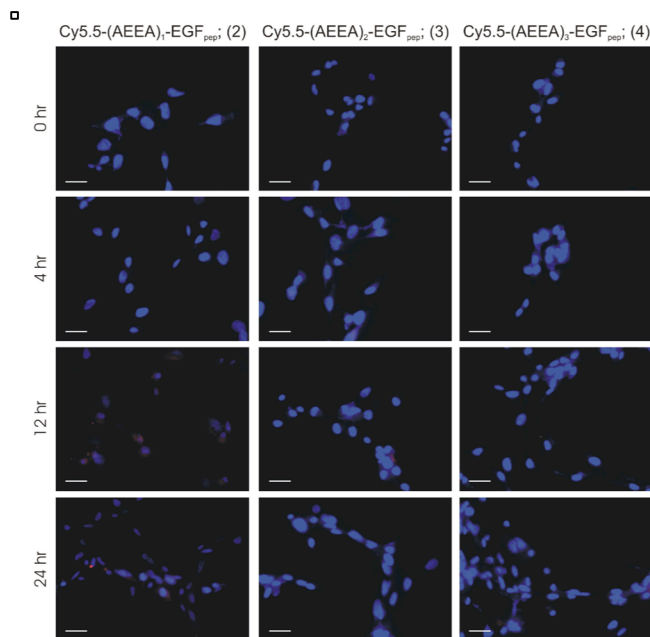


Fig. 3. No accumulation of peptides in cells lacking wild-type EGFR. U87-MG cells were incubated with 1 μ M compounds for the indicated times. Uptake of the compounds was assessed using epi-fluorescence microscopy. Representative images are shown. Images = 40X magnification. Scale bar = 50 μ m.

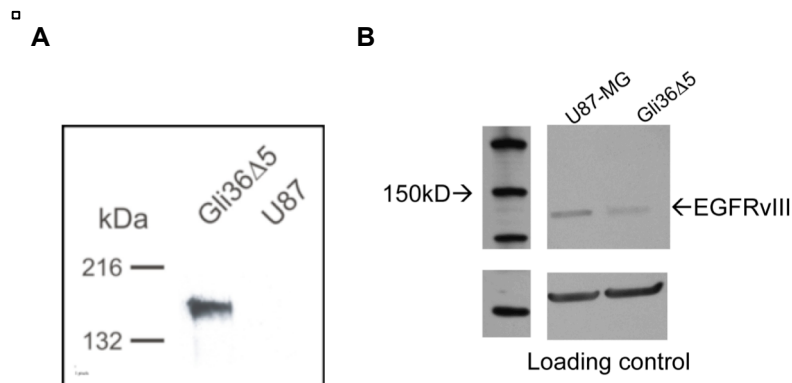


Fig. 4. Differential expression levels of EGFR and EGFRvIII. **A**, Relative levels of wild type EGFR expressed in Gli36Δ5 and U87-MG glioblastoma cell lysates using an antibody specific to wild type EGFR. **B**, Mutant EGFRvIII content in Gli36Δ5 and U87-MG tumor cells lines using an EGFRvIII specific antibody. β -actin was used as a loading

Consequently, we were able to determine the optimal peptide length and degree of functionalization of each linker length. We were also able to create NIR-conjugated peptides that targeted with variable receptor affinities and demonstrated that the linker length affected uptake and accumulation using the wild type EGFR specifically.

During the time period covered in this progress report, we were able to characterize a series of engineered *in vitro* cancer signature glioma cell lines as outlined in **Milestone 3**. Rat glioma cells (9L) were stably transfected with pcDNA3.1 plasmids containing the full length coding region of the human EGFR or the human transferrin receptor (TfR). To create a

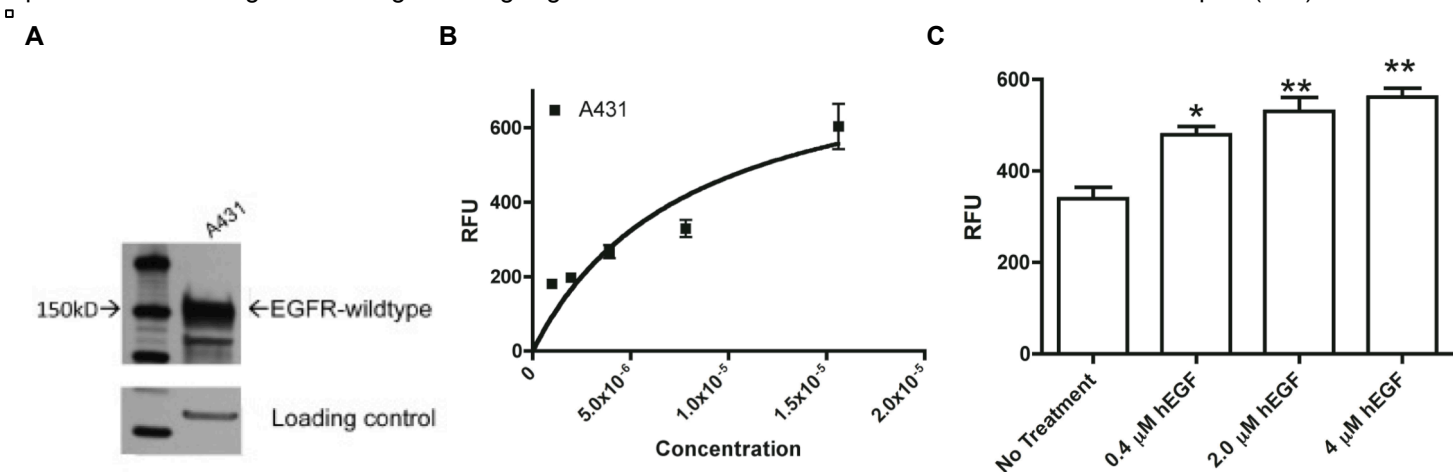


Fig. 5. A, Expression levels of the wild type EGFR determined by Western blot in the A431 cell lines. Loading control was determined by blotting against β -actin. **B**, Saturation binding assay. Compound **2** was incubated with A431 cells at increasing concentrations for 1 hour, washed, and cell associated Cy5.5 was quantified. The K_d determined for the A431 cell line was $8.0 \pm 3.0 \mu$ M. **C**, Cell uptake of compound **2** in the presence of hEGF ligand. A431 cells were plated in 96-well plates and were treated with 1 μ M of Cy5.5-EGFpep in the presence of increasing amounts of hEGF (or no treatment) for 90 minutes. Uptake increased with increasing EGF stimulation. These data suggest that uptake of labeled ligand is specific to EGF receptors. *, $P > 0.01$; **, $P > 0.001$.

cell line overexpressing both receptors, 9L cells overexpressing TfR were stably transfected with pcDNA3.1-EGFR and selected using antibiotics. Western blotting was used to show the relative expression of each human receptor protein in engineered cell lines (**Fig. 6A**). Immunofluorescence staining localizes the receptors both at the cell membrane (arrows) and within the cytoplasm of the cells (**Fig. 6B**). We also examined the expression of EGFR and TfR in the human glioma cell lines, Gli36Δ5 and U87, described in detail above. □

To prepare the β -galactosidase (β -gal) complexes, we linked biotinylated EGF for the EGFR to biotinylated β -gal using fluorophore-conjugated streptavidin. Ligand, linker, and reporter fragment were mixed in a molar ratio 1:1:3 at room temperature for 1 hr. Excess D-biotin was added to block any remaining unbound streptavidin sites. In the case of control assays, untargeted reporter complex was prepared with D-biotin, in place of the ligand, mixed with linker and reporter fragment in a molar ratio 1:1:3.

In our studies, complementation of the β -gal subunits involves a multiplicity of molecules; thus, tracking the uptake and accumulation of each single targeted-complex is necessary to evaluate the bioavailability of the imaging agents as outlined in **Milestone 4**. We used fluorescence microscopy to follow the spatial and temporal distributions of targeted- β -galactosidase subunits during internalization of the target, EGFR. Full-length EGF, the targeting moiety, was linked to either the α -4 subunit (EGF- α -4) or ω subunit (EGF- ω) of β -gal. Human glioblastoma cells, Gli36Δ5, overexpressing the EGFR were incubated for 4 hrs at 37 °C with each complex singly. An intrinsic fluorophore conjugated to the linker allowed us to examine the sub-cellular localization of each targeted-complex. As expected, cells that overexpress the EGFR bind and take up the EGF-targeted β -gal subunits. The targeted-complexes can be found throughout the functional EGFR internalization pathway, primarily in early endosomes (**Fig. 7A**) and lysosomes (**Fig. 7C**). Very little of either EGF-targeted complex was found in late endosomal vesicles (**Figure 7B**). Addition of the EGFR-targeted complexes did not adversely

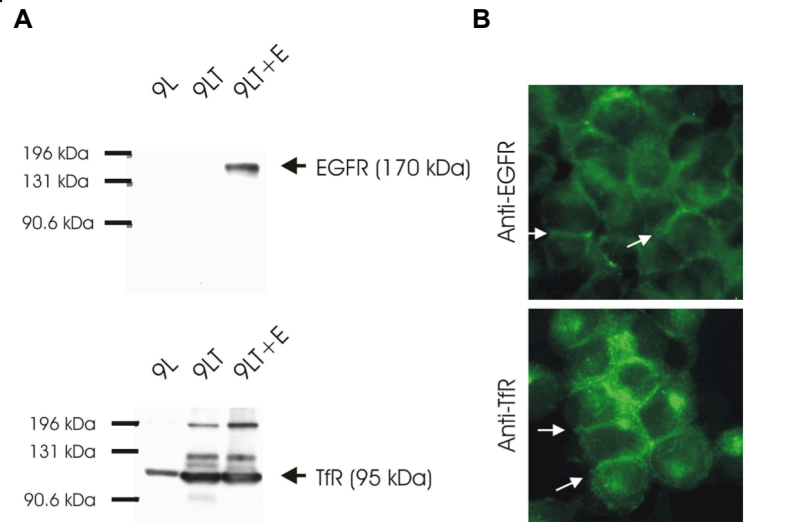


Fig. 6. Genetically engineered rat glioma, 9L, cell lines overexpressing EGFR and TfR. **A**, Western blot analysis of cell lines overexpressing none (9L), one (9LT) or two (9LT+E) human cell surface receptors. **B**, The overexpressed receptors are localized to the cell membrane (white arrows) and within the cytoplasm after staining with either anti-EGFR or anti-TfR.

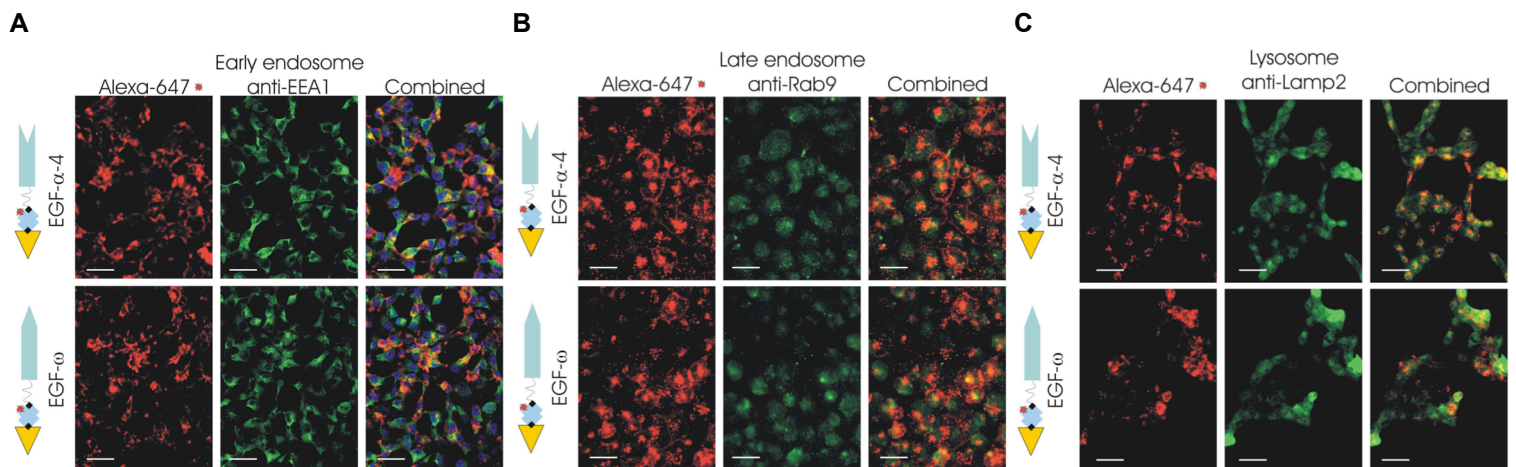


Fig. 7. EGFR-targeted split- β -galactosidase pairs co-localize in vesicles in the endosomal pathway. Gli36Δ5 cells were incubated with EGF ligand-complex (1 nMole) for 1 hr at 37 °C. The cells were then fixed with 4% paraformaldehyde, rinsed with PBS, and blocked with 1% host serum for 30 min at room temperature. Coverslips were incubated with primary antibody against EEA1 (**A**), Rab9 (**B**), or Lamp-2 (**C**) at room temperature for 2 hrs. The cells were counterstained with DAPI to visualize the nuclei, mounted, and observed using confocal microscopy. Images = 40X magnification. Scale bar = 50 μ m.

affect the viability of the cells as assayed by trypan blue staining (**not shown**).

Next, we examined whether concomitant incubation of cells with both targeted β -gal subunits altered either the uptake or location of the targeted-complexes (**Fig. 8A**).

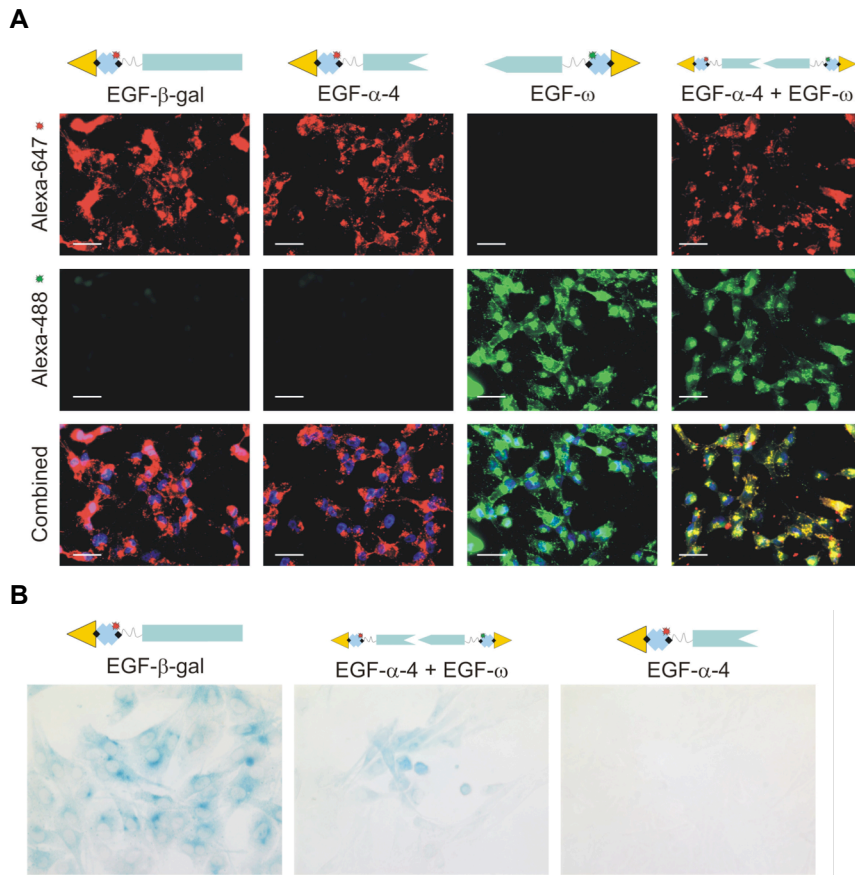


Fig. 8. *In vitro* complementation of targeted split- β -galactosidase pairs. **A**, Immunofluorescence of Gli36 Δ 5 cells incubated with full length β -gal or complementing pairs of β -gal subunits. Co-localization is seen by overlay of red and green to produce yellow. **B**, Cells incubated with either targeted-full length β -gal or complementing pairs retained enzymatic activity as seen after X-gal staining. Images = 40X magnification. Scale bar = 50 μ m.

complex. As a final control, uptake specificity of EGF- α -4 or EGF- ω was competitively inhibited in the presence of increasing concentrations of free EGF ligand (**Fig. 10**). Uptake of EGF- α -4 is dramatically decreased with both a 1:1 (200 nM) and 1:5 (1000 nM) ratio of complex to free ligand as visualized by fluorescence microscopy (**Fig. 10A**). EGF- ω is also inhibited, but not to the same extent as EGF- α -4. The relative fluorescence of each treatment regime was quantified and graphed. EGF- α -4 binding was reduced by 68% at the highest concentration of free ligand and EGF- ω was reduced by approximately 40% (**Fig. 10B**).

Several different combinations of complementing pairs of β -gal were examined for uptake and enzymatic activity as proposed in **Milestone 5**. The most robust pair to date is the α -4 and ω combination. The α -4 and 1- ω pair also demonstrated relatively strong enzymatic activity, but staining took longer the optimal 18 hrs. The only inefficient pair was the α -1 and 1- ω pair. This is not unexpected since the α -1 fragment is very small and rapidly diffuses away, out of the cellular compartments where complementation could be optimized. In the short term, we will move forward into *in vivo* studies with the α -4 and ω complementing pair.

Gli36 Δ 5 cells were incubated for 4 hrs at 37 °C with EGF- β -gal, EGF- α -4 singly, EGF- ω singly, or the combination of EGF- α -4 and EGF- ω . In this case, the EGF- α -4 complex was labeled with Alexa 647 fluorophore and the EGF- ω complex was labeled with Alexa 488 fluorophore to distinguish between the two complexes. EGFR-targeted α -4 and ω complexes each sequestered in a manner similar to that of targeted-full length β -gal. The combination of both targeted-complexes did not negatively impact the uptake of either complex. The targeted-complexes co-localized to the same location predominately, although EGF- α -4 was observed in disparate locations approximately 20% of the time as measured by relative fluorescence densitometry. Enzymatic activity was visualized by overnight X-gal staining (**Fig. 8B**).

Incubation of EGF- α -4 or EGF- ω with its non-targeted, complementing partner, i.e. B- ω or B- α -4, respectively, further demonstrated the specificity of the targeting moiety to drive the uptake and accumulation of the complex (**Fig. 9**). Non-targeted complementing fragments were not internalized as shown by a lack of intrinsic fluorescent signal; also, the presence of the non-targeted fragment did not inhibit the uptake of the targeted-

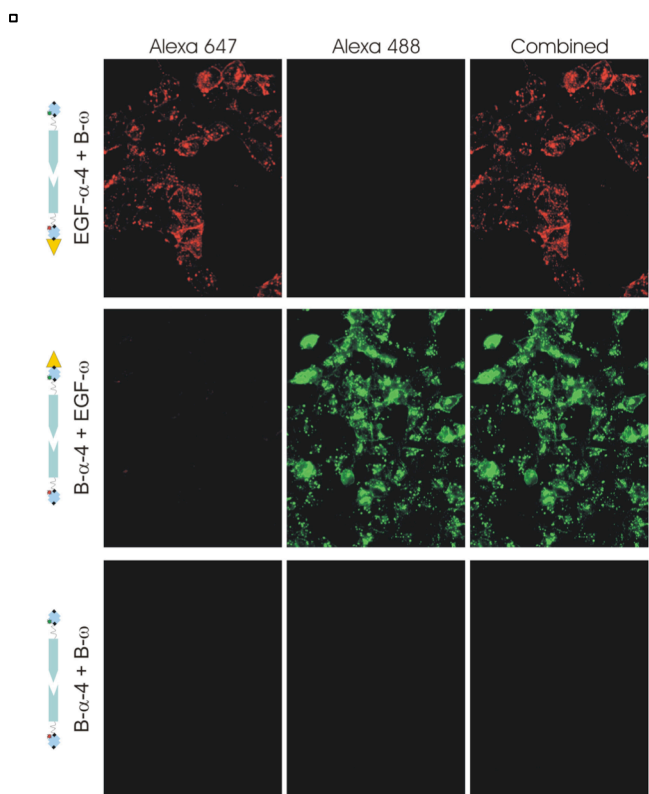


Fig. 9. Non-targeted β -galactosidase fragments do not accumulate within the cells. Gli36 Δ 5 cells incubated with 1 nM targeted-subunit simultaneously with the non-targeted complementing subunit for 1 hr at 37C only accumulated the targeted-subunit as observed by confocal microscopy. Images = 40X magnification.

KEY RESEARCH ACCOMPLISHMENTS

The following lists the key research accomplishments emanating from this research during this period:

- Synthesis of NIRF-EGF peptide probe (EGF-Cy5.5) with high affinity for the EGFR and specific accumulation in cells overexpressing EGFR.
- Demonstration that mutant EGFR, EGFRvIII, is not required to effectively image cancer cells *in vitro*.
- Characterization of linker length on uptake and accumulation of EGF-Cy5.5 using kinetic dissociation assay and immunofluorescence microscopy.
- Creation of genetically engineered rat glioma cell lines overexpressing no human receptors, one human receptor, or combination of two human receptors.
- Evaluation of EGFR and TfR pattern of overexpression in human glioma cell lines, Gli36 Δ 5 and U87.
- Self-assembly of targeted β -gal complexes.
- Track uptake and accumulation of EGFR targeted-complex into the endosomal pathway.
- *In vitro* complementation of targeted split- β -galactosidase pairs.
- Recognition that the optimal complementing β -gal pairs, α -4 and ω , are the most robust pair to move forward into *in vivo* animal tumor model studies.

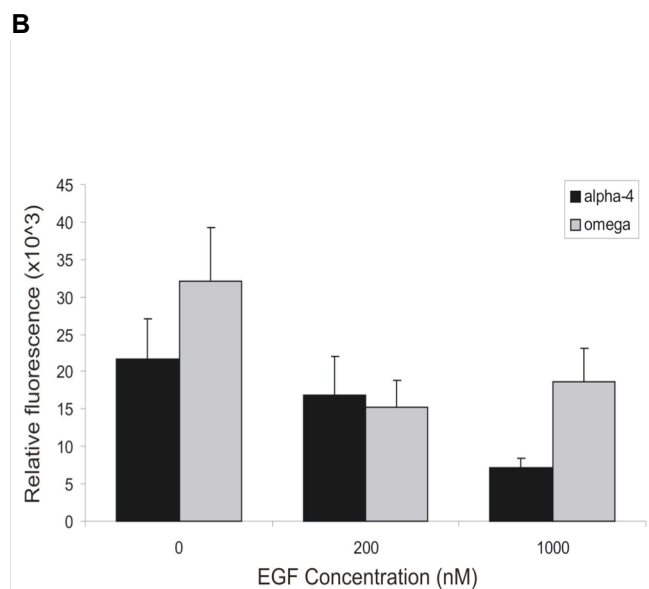
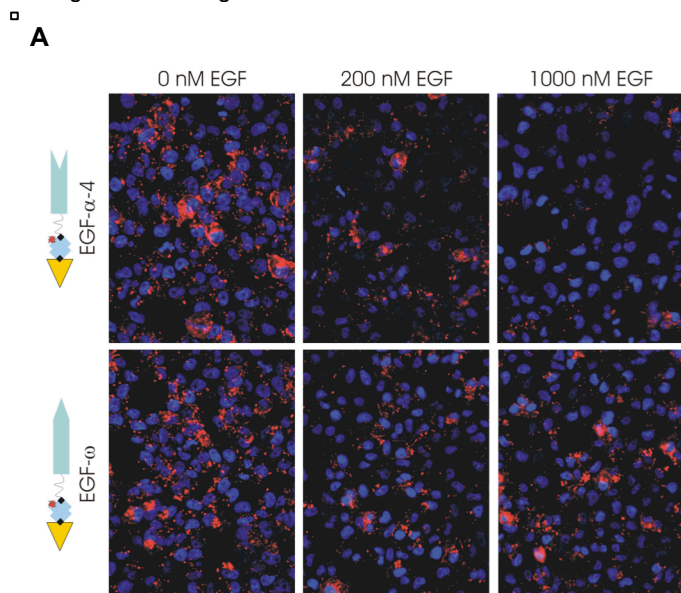


Fig. 10. Targeted β -galactosidase fragment uptake is specific for the EGF receptor. Gli36 Δ 5 cells were incubated with 1 nM targeted-subunit simultaneously with increasing concentrations of free EGF for 1 hr inhibited uptake of the targeted complex. Fluorescence was quantified and graphed to demonstrate the reduction in complex binding. Images = 40X magnification.

REPORTABLE OUTCOMES

Original research papers currently in preparation:

Broome, A-M.*; Ramamurthy, G.; Lavik, K.; Liggett, A.; Verma, A.; & Basilion, J.* (2012) Optical imaging of targeted beta-galactosidase complementation in brain tumor cell lines. *Nature Biotechnology*. (*corresponding author) Impact factor: 31.09

Invited lectures:

- Molecular imaging gets personal: The future of personalized diagnostics and therapeutics. (2012) *Wayne State University School of Medicine Radiology Grand Rounds*.
- Molecular imaging gets personal: The future of personalized diagnostics and therapeutics. (2011) *Malone University Alpha Gamma Chapter Induction Ceremony*.
- Molecular imaging: The future of personalized medicine. (2011) *CWRU School of Medicine Radiology Grand Rounds*.
- Novel molecular imaging platforms. (2011) *University of Akron Chemistry Seminar Series*.
- Molecular imaging gets personal: targeting the cancer signature. (2011) *MUSC Center for Biomedical Imaging Seminar Series*.
- Imaging the tumor microenvironment with novel bio-molecule constructions. (2011) *Tumor Microenvironment and Metastasis Focus Group*.
- Building targeted-split enzyme nanomolecule complexes using directed self-assembly. (2011) *Foundations of Nanoscience: Self-Assembled Architectures and Devices, 8th Annual Meeting*.

Oral presentations at international conferences:

Broome, A-M.; Ramamurthy, G.; Lavik, K.; Liggett, L.A.; & Basilion, J.P. (2011) Targeting split-enzyme reporter fragments to achieve chemical resolution for molecular imaging. *SPIE BiOS: Biomedical Optics Symposium*.

Poster presentations at international conferences and workshops:

- **Broome, A-M.**; Ramamurthy, G.; Lavik, K.; Pinter, M.; Kinstlinger, I.; & Basilion, J.P. (2012) Targeted-split enzyme complementation to interrogate the cancer signature using molecular imaging. *World Molecular Imaging Congress*.
- **Broome, A-M.**; Ramamurthy, G.; Lavik, K.; Verma, A.; Pinter, M.; Basilion, J.P. (2012) Molecular imaging of the cancer signature using targeted-split enzyme complementation. *American Association for Cancer Research, 103rd Annual Meeting*.
- Verma, A.; Ramamurthy, G.; Chung, S.; **Broome, A-M.**; and Basilion, J.P. (2011) Targeted-enzyme complementation to image cancer receptors. *BMES Annual Meeting*.
- **Broome, A-M.**; Ramamurthy, G.; Lavik, K.; Verma, A.; & Basilion, J.P. (2011) Targeted-split enzyme nanomolecule complexes for molecular imaging of the coordinated expression of cell surface receptors in glioblastomas. *World Molecular Imaging Congress. Best Poster in Category*.
- Agnes, R.S.; **Broome, A-M.**; & Basilion, J.P. (2011) Differential fluorescence molecular imaging of EGFR in brain tumors. *World Molecular Imaging Congress*.
- Agnes, R.S.; **Broome, A-M.**; & Basilion, J.P. (2011) Targeted, non-invasive optical imaging agent for fluorescence molecular tomography studies. *American Peptide Society, 22nd American Peptide Symposium*.
- Ramamurthy, G.; **Broome, A-M.**; Lavik, K.; Verma, A.; & Basilion, J.P. (2011) Imaging the coordinated expression of cell surface receptors in glioblastomas using a novel multi-functional enzyme reporter complementation complex. *American Association for Cancer Research, 102nd Annual Meeting*.

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Associate Professor
Director of Molecular Imaging, Center for Biomedical Imaging
Director of Small Animal Imaging, Hollings Cancer Center
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CONCLUSION

Targeted-reporter platforms have real utility for imaging the multi-step progression of cancer growth that requires the coordinated overexpression of cell surface biomarkers. The development of these platforms to investigate molecular signatures associated with disease creates the next frontier in *in vivo* imaging. The exponential number of genomic marker sets that are considered diagnostic or predictive of disease states underscores the importance of imaging molecular signatures. By exploiting multi-marker imaging, we ultimately seek to image combinations of biomarkers that will uniquely identify cancers from normal tissue and report on the biochemical status of these cells. These expression patterns can thus be indicative of the type, stage, or severity of disease. The application of imaging molecular signatures is therefore critical for cancer and disease detection.

Our research utilizes inactive subunits of an image-able enzyme that are driven to complementation by targeting specific biomarkers on the surface of cells. By linking a reporter fragment to a targeting moiety, this approach provides a cellular resolution that is far more precise than the physical/ anatomical-based resolution currently employed. The pioneering utility of the technique significantly increases specificity, decreases background artifacts, and, combined with a cancer systems approach, promotes our ability to interrogate the status of cells rather than just the presence of cancer biomarkers. In the studies described here, we will utilize a bi-complementation strategy.